Food Chemistry 126 (2011) 664–672

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Differentiation of blossom and honeydew honeys using multivariate analysis on the physicochemical parameters and sugar composition

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ARTICLE INFO

Article history:
Received 12 May 2010
Received in revised form 22 September 2010
Accepted 2 November 2010

Keywords:
Honeydew honey
Blossom honey
Physicochemical and palinological parameters
Sugar composition
Multivariate analysis

ABSTRACT

Multivariate analysis was applied on physicochemical parameters (moisture, water activity, electric conductivity, colour, hydroxymethyl furfural, acidity, pH, proline, diastase and invertase), sugar composition (fructose, glucose, sucrose, maltose, isomaltose, trehalose, turanose and melezitose) and palinological parameters determined in blossom and suspected honeydew honeys in order to differentiate them. The majority of the physicochemical, sugar composition and palinological parameters evaluated presented significant differences in the mean values between the suspected honeydew and blossom honeys, with the exception of moisture, water activity, diastase, fructose and maltose. Blossom honey samples tend to differentiate from the suspected honeydew honeys after applying factor analysis on the physicochemical parameters and sugar composition. Stepwise linear discriminant analysis allows the correct classification of all blossom honeys, and only one honeydew honey was erroneously included as blossom honey. So, the use of multivariate analysis on physicochemical parameters and sugar composition can be a useful tool to differentiate these types of honeys.

1. Introduction

Honey is a very complex product composed of major compounds including monosaccharides such as glucose and fructose and minor components such as amino acids, enzymes, vitamins and minerals. Honey has been used since ancient times mainly as a sweetening agent. Besides, a wide and diverse range of therapeutic activities such as its antimicrobial and antioxidant properties, stimulating the healing of wounds and burns and gastric ulcers treatment (Ouchemoukh, Louaileche, & Schweitzer, 2007) have also been attributed to the use of honey.

Blossom honey is produced by honeybees from the nectar of blossoms, while honeydew honey is prepared from the secretions of living parts of plants or excretions of plant sucking insects on the living part of plants. According to the European Legislation (European Economic Community, 2002), blossom honeys have electric conductivity values below 0.80 ms/cm, while the honeydew honeys exceed this value. However, there are many exceptions to this rule. Some monofloral honeys such as those from chestnuts, strawberry plants, heather, eucalyptus, lime, manuka, tea tree or jelly bush honeys, regarded as blossom honeys, often have electric conductivity values above 0.8 ms/cm (European Economic Community, 2002; Persano Oddo & Piro, 2004).

The application of multivariate analysis to the general physicochemical parameters, minerals, trace elements, and sugars has been used to differentiate types of monofloral honeys, honeydew and blossom honeys over the last few decades (Marini, Magrì, Balestrieri, Fabretti, & Marini, 2004; Terrab, Díez, & Heredia, 2002; Terrab, González, Díez, & Heredia, 2003). However, Díez, Andrés, and Terrab (2004) could not confirm the origin (blossom or honeydew honeys) in all the 152 honeys from Morocco using the physicochemical parameters (pH, mineral content, electric conductivity, sugars and colour). The different kinds of honeys produced in different regions could explain these discrepancies.

Currently, there is no evidence of the presence of honeydew honeys in Tenerife. However, there are certain doubts whether some honey samples, commonly considered as blossom honeys, could in fact be honeydew honeys. These honeys did not show the characteristics of other known monofloral or multifloral honeys from Tenerife. They have typical malted, caramelized and salty flavours, common with honeydew honeys produced in other Spanish and European regions. The whiteflies *Aleurodicus dispersus* Russell and *Lecanoideus floccissimus* M. present in the Canary Islands, which affect different crops and ornamental plants, could be

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doi:10.1016/j.foodchem.2010.11.003

involved in the origin of these honeys. These insects arrived in the Canary Islands in 1991 *via* ornamental palm trees from the Caribbean. Since then they have been affecting different crops and ornamental plants. These insects produce typical white cotton-like secretions and abundant molasses that marks and damage the host leaves and surrounding areas. Beekeepers have observed bees working in free sugars on leaves from affected plants and on the ground, but this relationship has still not been entirely demonstrated, so further analyses are necessary in this field (Bentabol-Manzanares, Hernández-García, González-Rodríguez, & Santos-Vilar, 2008).

This study was undertaken to find out differences between blossom and suspected honeydew honeys according to their physicochemical and palinological characteristics. Statistical multivariate analysis was applied on their physicochemical and sugar composition in order to classify both types of honey samples.

2. Material and methods

2.1. Honey samples

A total of 77 samples of blossom ($n = 53$) or suspected honeydew ($n = 24$) honeys were freshly strained from the combs in the years 2005–2008. The criterion for differentiating both types of honeys was the electric conductivity value (EC), which has a high discriminatory power to differentiate honeydew and blossom honeys (European Economic Community, 2002). Thus, the honey samples were divided into two groups: (1) EC $\leqslant$ 0.80 mS/cm, which were the blossom honeys; and (2) EC > 0.80 mS/cm, which could be honeydew honeys (suspected honeydew honeys). All the honey samples were frozen at −18 °C until analysis. Maturity and deterioration criteria indicated that the quality of the honeys was good, and no samples showed signs of fermentation.

2.2. Reagents and solutions

Ultra pure water (Milli Q, Millipore Corp, Bedford, MA, USA) was purchased for chromatographic use. HPLC grade acetonitrile was purchased from Scharlau (Barcelona, Spain). Standards of glucose, fructose, sucrose, mannose, maltose, trehalose, isomaltose, erlose and melezitose came from Fluka (Buchs, Switzerland) and turanose from Sigma (Sigma–Aldrich, Madrid, Spain). Stock solutions (2 g/l) were prepared for dissolution in ultrapure water and were stored at 4 °C. Carrez I and II, acetic acid (glacial), formic acid (98%), proline standard, ninhydrin solution (3%) were purchased from Panreac (Barcelona, Spain). Phadebas for diastase determination was purchased from Magle (Lund, Sweeden). *p*-nitrophenyl-α-D-glucopyranoside was purchased from Sigma (Sigma–Aldrich, Madrid, Spain). All the other chemicals and reagents used were analytical grade.

2.3. Physicochemical analysis

All the physicochemical parameters studied were determined according to the standardised methods proposed by International Honey Commission, 2009 Moisture was determined by Atago Model 1T Abbé-type refractometer at 20 °C (Atago Co, Tokyo, Japan). Water activity (a_w) was determined at 25 ± 0.02 °C as the vapour pressure at the dew point using a Thermoconstanter instrument (Novasina ms-1, Switzerland). Electrical conductivity was measured at 20 °C in a 20% (w/v) solution of honey in ultrapure water with Radiometer Meterlab (Brønshog, Denmark CDM conductimeter) and the results were expressed as mS/cm. Colour was determined using a Pfund colour grader Koehler (New York, USA) and the results are expressed as mm Pfund. Hydroxymethyl furfural (HMF) was determined by a colourimetric method after clarifying the samples with Carrez reagents (I and II) and addition of sodium bisulphite. Absorbance was measured at 284 nm and 336 nm in an UVmini-1203 Shimadzu spectrophotometer (Shimadzu Europe GmbH, Duisburg, Germany) and results were expressed as mg/kg. Free acidity was determined by titration with 0.05 M NaOH to pH 8.30 and the results were expressed as meq/kg. The pH was measured in a solution of 10 g of honey in 75 ml ultrapure water with a pH metre (radiometer pHM 220). Proline content was determined by the measurement of the absorbance at 510 nm of the resulting product between proline and ninhydrin in an acidic medium. The diastase activity was measured using the Phadebas method for α-amylase. Phadebas is a synthetic reagent which produces a blue colour when it is hydrolysed by the diastase. The absorbance at 620 nm is directly proportional to the diastase activity in the honey sample. Results are expressed in Gothe units per gram of honey, defined as that amount of the enzyme which will convert 0.01 g of starch into the prescribed end point in 1 h at 40 °C under test conditions. Invertase activity was spectrophotometrically measured with 4-nitrophenyl-α-D-glucopyranoside and the results are expressed in international units (IU).

2.3.1. Sugar determination

About 0.5 g of honey was weighed directly into polypropylene tubes and mixed with 10 ml of 60% methanol. Afterwards, a millilitre of the dissolution was filtered through a 0.45 μm filter GHP (Waters, Millford, MA, USA) prior to HPLC analysis.

The determination of sugars was performed with a Waters 2690 high-performance liquid chromatograph equipped with a differential refractive index (DRI) detector (Waters model 2414). The separation was performed by using a Waters Carbohydrate Analysis column (3.9 × 300 mm) with a particle size diameter of 10 μm, equipped with a Waters Carbohydrate Carbo™ 4 μm guard column. The column was kept at 25 °C throughout the analysis. The HPLC pumps, autosampler, column oven and DRI detector were monitored and controlled using the Millennium^{32} system. The mobile phase was composed of 80% acetonitrile in water. The injection volumes of the samples were 25 μl, with a flow rate of 2 ml/min. The HPLC sample peaks were identified by comparing the retention times obtained from standards. The honey samples were also spiked with standards in order to verify the identity of the chromatographic peaks. Duplicate injections were performed and average peak areas were used for the peak quantification.

2.3.2. Pollen analysis

Pollen analysis was carried out using the methods established by the International Commission of Bee Botany reported by Louveaux, Maurizio, and Vorwohl (1978) using a Olympus CH 30 Microscope coupled to a digital TV Camara JVC Digital TK-C1381. The family and pollen type as well as the number of pollen grains and honeydew elements/number of pollen grains from nectariferous species (HDE/NPGN) were determined. The frequency of the different pollen types is divided into the following four classes: as predominant pollen (>45%); secondary pollen (16–45%); minor important pollen (3–15%); minor pollen (1–3%); and sporadic pollen (<1%).

2.4. Statistics

All the statistics were performed by means of the SPSS version 17.0 software for Windows (SPSS Inc. Chicago, IL). Mean values obtained for the variables studied in the different groups were compared by One-Way ANOVA (Duncan's multiple range) assuming there were significant differences among them when the statistical comparison gave $p < 0.05$. Simple linear correlation analysis was used to indicate the level of the correlation and the strength of

Table 1
Pollen types and their frequency in honeydew honeys ($n = 24$) and blossom honeys ($n = 53$). *P*, predominant pollen (>45%); *S*, secondary pollen (16–45%); *M*, minor important pollen (3–15%); *T*, minor pollen (1–3%); *R*, sporadic pollen (<1%); *N*, nectariferous plant; NL, nectarless plant.

Family	Pollen type		Honeydew honey				Blossom honey			
			P (%)	*S* (%)	*M* (%)	*T* (%)	*P* (%)	*S* (%)	*M* (%)	*T* (%)
Aizoaceae	*Messembryanthemum crystallinum*	N	5	–	5	11	20	10	20	18
Amaranthaceae	*Achyranthes aspera*	N	–	–	26	32	8	15	30	27
Anacardiaceae	*Schinus molle*	N	–	5	21	21	–	3	10	30
Apiaceae	*T. Ferula linkii*	N	–	5	–	–	–	2	5	–
	T. Foeniculum vulgare	N	–	21	16	26	3	10	30	17
Aquifoliaceae	*Ilex canariensis*	N	–	–	–	16	–	–	18	18
Asteraceae	*Argyranthemum* sp	N	–	5	5	5	2	3	13	22
	Artemisia thuscula	NL	–	–	–	–	–	–	–	15
	Calendula arvensis	N	–	–	16	47	–	3	30	25
	Carlina salicifolia	N	–	–	16	26	–	–	8	25
	Carlina xeranthemoides	N	–	–	5	5	–	2	2	–
	Kleinia neriifolia	N	–	–	5	26	–	–	–	5
	T. Dittrichia viscosa	N	–	5	11	16	–	5	23	33
	T. Galactites tomentosa	N	–	–	26	37	–	–	18	35
	T. Sonchus	N	–	5	32	26	2	2	27	42
Boraginaceae	*Echium leucophaeum*	N	–	–	5	–	2	8	3	2
	Echium plantagineum	N	5	32	21	16	32	32	37	–
	Echium virescens	N	5	–	26	11	27	12	17	7
Brassicaceae	*Descurainia bourgeauana*	N	–	–	26	16	3	10	27	18
	T. Brassica (Hirschfeldia incana)	N	–	16	37	37	12	22	50	13
Cactaceae	*Opuntia ficus indica*	N	–	–	5	–	–	–	–	7
Campanulaceae	*Campanulaceae*	N	–	–	5	–	–	–	2	2
	Canarina canariensis	N	–	–	–	–	–	–	2	7
Caprifoliaceae	*Viburnum tinus ssp. rigidum*	N	–	–	5	5	–	–	5	10
Carycaceae	*Caryca papaya*	N	–	–	–	–	–	–	5	2
Caryophyllaceae	*Silene sp (S. vulgaris)*	N	–	–	–	–	–	–	2	7
Cistaceae	*Cistus symphytifolius*	NL	–	–	21	42	5	8	43	28
Convolvulaceae	*Convolvulus althaeoides / C. floridus*	N	–	–	5	21	–	–	2	17
Crassulaceae	*Aeonium urbicum*	N	21	–	26	11	3	2	38	30
Cucurbitaceae	*Bryonia verrucosa*	N	–	–	5	21	–	–	2	7
	Cucurbita maxima	N	–	–	–	5	–	–	2	2
Dipsacaceae	*Pterocephalus lasiospermus*	N	–	–	16	16	–	–	12	15
Ericaceae	*Erica arborea*	N	11	21	32	16	13	25	22	17
Euphorbiaceae	*Euphorbia sp (E. obtusifolia)*	N	–	–	5	16	–	–	10	25
	Ricinus communis	NL	–	–	26	21	–	–	13	17
Fabaceae	*Aspalthium bituminosum*	N	–	–	–	16	–	2	30	28
	Lotus campylocladus	N	–	–	–	–	2	10	8	7
	Lotus sessilifolius	N	–	–	11	–	2	2	12	13
	Parkinsonia aculeata	N	–	–	5	–	–	7	3	2
	Spartocytisus supranubius	N	11	–	16	–	40	–	–	–
	T. Chamaecytisus proliferus	N	5	11	32	11	13	22	23	2
	Trifolium sp	N	–	–	–	–	–	–	–	5
	Vicia sp (V. benghalensis)	N	–	–	5	11	–	2	5	18
Fagaceae	*Castanea sativa*	N	84	5	11	–	37	20	23	3
Globulariaceae	*Globularia salicina*	N	–	–	16	26	–	2	2	7
Hypericaceae	*Hypericum* sp	NL	–	–	–	–	–	3	5	20
Lamiaceae	*Lavandula canariensis*	N	–	–	–	–	–	–	–	18
	Nepeta teydea	N	–	–	–	11	–	–	5	8
	Origanum vulgare ssp. virens	N	–	–	16	21	–	–	8	10
	Sideritis oroteneriffae	N	–	–	–	–	–	2	–	10
	T.Origanum virens (Bystropogon origanifolius)	N	–	–	–	–	2	–	3	10
Lauraceae	*Persea americana*	N	–	5	21	21	–	20	23	17
Liliaceae	*Asparagus* sp	N	–	–	16	5	–	2	8	20
	Liliaceae sp 1	N	5	–	5	11	–	2	3	8
	Liliaceae sp 2	N	–	5	5	16	–	2	7	8
	Pancratium canariensis	N	–	–	–	–	–	–	2	5
	Phoenix canariensis	NL	32	21	21	16	3	5	18	23
Mimosaceae	*Acacia cyanophylla*	N	–	–	–	5	–	–	2	20
Myricaceae	*Myrica faya*	NL	–	–	5	11	–	2	13	15
Myrtaceae	*Eucalyptus camaldulensis*	N	–	5	–	–	–	5	8	15
	Eucalyptus globulus	N	16	11	32	11	–	10	28	18
Oleaceae	*Olea europaea*	NL	–	–	–	11	–	–	–	8
Oxalidaceae	*Oxalis pes0caprae*	N	–	–	16	47	–	5	32	22
Papaveraceae	*Papaver* sp *(P. somniferum)*	NL	–	–	–	26	–	–	–	7
Pinaceae	*Pinus canariensis*	NL	–	–	5	–	–	–	–	3
Plantaginaceae	*Plantago lagopus*	NL	–	11	26	16	–	2	7	10
Poaceae	*Zea mays*	NL	–	–	–	5	–	–	3	10
Polygonaceae	*Polycarpaea divaricata / P. nivea*	N	–	–	–	–	–	3	7	7
	Rumex sp *(R. lunaria)*	NL	–	–	26	32	–	5	48	18
Resedaceae	*Reseda luteola*	N	–	5	–	5	3	3	17	17
Rhamnaceae	*Rhamnus* sp	N	–	–	5	5	5	2	15	17
Rosaceae	*Marcetella moquiniana*	N	–	–	11	16	–	3	3	7

Table 1 (*continued*)

Family	Pollen type		Honeydew honey				Blossom honey			
			P (%)	*S* (%)	*M* (%)	*T* (%)	*P* (%)	*S* (%)	*M* (%)	*T* (%)
	Prunus dulcis	N	–	5	16	11	–	3	15	32
	Prunus persica	N	–	–	–	11	2	5	5	–
	Rubus ulmifolius	N	5	32	53	5	5	5	27	20
Rubiaceae	*Plocama pendula*	N	–	–	5	–	–	–	8	12
Rutaceae	*Citrus sinensis / C. limon*	N	–	–	5	16	–	–	3	37
Salicaceae	*Salix canariensis*	N	–	–	5	–	2	8	12	17
Sapindaceae	*Cardiospermum grandiflorum*	N	–	–	–	5	–	–	–	12
Scrophulariaceae	*Scrophularia* sp	N	–	–	5	–	–	–	5	–
Thymelaeaceae	*Daphne gnidium*	N	–	–	–	16	–	–	–	5
Tropaeolaceae	*Tropaeolum majus*	N	–	–	47	11	8	18	3	15

the relationship between two variables. Factor analysis, using principal components as the method of factor extraction, was used to establish a more simplified view of the relationship among the physicochemical and sugar parameters analysed. Linear discriminant analysis (LDA) was applied to classify the honey samples into homogeneous groups established by the dependent variable.

3. Results and discussion

3.1. Pollen analysis

The results from the pollen analysis are briefly summarised in Table 1. All the honeys analysed could be considered as multifloral honeys according to the pollen contribution of the botanic species.

No honeys had sufficiently predominant pollen contents from anyone botanical specie to be considered as a monofloral honey. A high number of pollen types in both groups of honeys (24.8 ± 7.9 and 27.9 ± 7.0 different pollen types for blossom and honeydew honeys, respectively) were observed which constitutes the common spectra of the Tenerife honeys. Suspected honeydew honeys showed lower frequencies of major pollen types (>45%) than blossom honeys, which is due to the low number of pollen grains/g of honey. Therefore, these honeys are characterised by a wide variety of pollen types which is a typical characteristic of honeydew honeys Persano Oddo and Piro (2004). This is explained by the fact that the sugar source for the honey production by bees is extrafloral and as a consequence the bees do not bring big loads of pollen with these sugar secretions. Besides, a higher frequency of pollens from anemophilous botanical species is present in honeydew honeys. These pollens are transported by air to sugar secretions on the leaves and other parts of the plants and the bees then collect them along with the sugar to make the honeydew honey.

In general, the presence of pollens of nectarless and anemophylous plants in the honeydew honeys was higher than in the blossom honeys. Therefore, for example, 84% of the suspected honeydew honeys (versus 37% of blossom honeys) had palm tree pollen (*Phoenix canariensis*) which is a nectarless plant.

Many authors have reported that under microscopic examination honeydew honeys are characterised by the presence of abundant honeydew elements such as mould hyphae, fungal spores, mycelium or unicellular algae. Louveaux et al. (1978) established that the HDE/pollen ratio in this kind of honey must be higher or equal to three. However, Persano Oddo and Piro (2004) reported an HDE/NPGN ratio of 1.5 ± 1.2 (0.3–4) in 167 honeydew honeys from different places from Europe. The suspected honeydew honeys analysed here presented a medium HDE/NPGN ratio lower than 0.3. Therefore, our results contrast the results reported by other authors (Persano Oddo & Piro, 2004; Terrab et al., 2002). This could be due to the special edafoclimatic conditions of the Canary Islands, or to the season (summer) when these honeys are collected. These special characteristics can influence the sugar excrements produced by plagues.

3.2. Physicochemical parameters and sugar composition

Table 2 shows the mean ± standard deviation, maximum and minimum values of the physicochemical parameters analysed for the two groups of honey considered, blossom honeys (EC $\leqslant$ 0.8 mS/cm) and suspected honeydew honeys (EC > 0.8 mS/cm). As a first approach, One-Way ANOVA test was applied to look for significant statistical differences.

In general, the physicochemical parameters in both groups of honeys considered were according to international standards indicated for suspected honeydew honeys and blossom honeys, respectively. All the honey samples had values of the legal parameters within the established intervals (European Economic Community, 2002).

Most of the parameters evaluated a high power to discriminate between the suspected honeydew and blossom honeys, with exceptions of moisture, water activity and diastase. It is well known that these three parameters are related to the maturity and freshness of samples, and not to their botanic origin or type of honey. The suspected honeydew honeys had significantly higher values of acidity, pH, electrical conductivity, colour, hydroxymethyl furfural (HMF), proline, diastase and invertase activities, fructose, maltose, trehalose and isomaltose, and lower values of glucose, sucrose, turanose and melezitose than those included as blossom honeys. Acidity, pH, electrical conductivity and proline, invertase and glucose showed the highest discriminating power ($p < 0.001$) between both types of honeys. These results are in agreement with the physicochemical characteristics of honeydew honeys from different countries reported by different investigators. So, several investigators (Golob & Plestenjak, 1999; Ouchemoukh et al., 2007; Terrab et al., 2003; Vela, de Lorenzo, & Pérez, 2007) have found that honeydew honeys were generally characterised by higher electric conductivity, as well as a higher pH, acidity, ash content and dark colour, than blossom honeys. Therefore, this supports the hypothesis that the honeys with EC higher than 0.8 mS/cm could be honeydew honeys.

The moisture values obtained in this paper ranged between 15.5% and 18.9%. In accordance with our results, Gleiter, Horn, and Isengard (2006) did not find significant differences in the moisture and water activity between both types of honey. The water content and water activity were highly correlated in both the types of honey considered ($r = 0.710$ and $r = 0.870$ for blossom and honeydew honeys, respectively). When this correlation was represented differentiating both types of honeys (Fig. 1), it could be observed that there were no differences between both types of honeys analysed. This contrasts with results reported by other investigators (Abramovic, Jamnik, Burkan, & Kac, 2008; Gleiter et al., 2006) who observed that honeydew honeys had higher water activities than liquid blossom honeys with the same water content.

Table 2
Physicochemical parameters and sugar composition in the types of honeys analysed, including the results of the One-Way ANOVA test for the comparison between the mean values.

	Honeydew honey $\bar{X} \pm$ SD (min–max)	Blossom honey $\bar{X} \pm$ SD (min–max)	p[a]
Moisture (%)	16.98 ± 0.77 (15.70–18.60)	16.73 ± 0.60 (15.50–18.90)	0.127
Water activity	0.59 ± 0.01 (0.57–0.61)	0.58 ± 0.01 (0.56–0.61)	0.205
Electrical conductivity (mS/cm)	1.21 ± 0.33 (0.83–2.09)	0.47 ± 0.17 (0.20–0.80)	**0.000**
Colour (mm Pfund)	127.8 ± 22.0 (71.0–150.0)	102.8 ± 32.7 (39.0–150.0)	**0.001**
HMF (mg/kg)	10.84 ± 6.48 (0.00–28.40)	7.67 ± 6.00 (0.70–26.00)	**0.040**
Acidity (meq/kg)	34.65 ± 10.57 (8.90–48.80)	24.44 ± 5.23 (15.50–41.80)	**0.000**
pH	4.58 ± 0.69 (3.87–6.91)	3.96 ± 0.28 (3.52–4.70)	**0.000**
Proline (mg/kg)	1069 ± 284 (664–1689)	602 ± 140 (310–1057)	**0.000**
Diastase (°Gothe)	16.74 ± 6.64 (4.70–25.80)	16.02 ± 5.62 (8.90–35.90)	0.956
Invertase (IU)	9.76 ± 2.99 (4.40–13.71)	7.15 ± 2.37 (3.90–16.41)	**0.000**
Fructose (%)	39.64 ± 1.71 (36.00–42.90)	39.54 ± 1.20 (35.90–42.10)	0.756
Glucose (%)	31.19 ± 2.33 (26.60–34.30)	33.29 ± 1.96 (29.20–38.70)	**0.000**
Sucrose (%)	0.50 ± 0.56 (0.05–1.87)	0.95 ± 0.65 (0.05–3.33)	**0.005**
Maltose (%)	5.52 ± 0.89 (3.85–6.74)	5.25 ± 0.88 (2.33–7.00)	0.222
Trehalose (%)	1.89 ± 0.42 (1.22–2.85)	1.67 ± 0.28 (1.15–2.38)	**0.008**
Turanose (%)	1.28 ± 0.25 (0.71–2.20)	1.46 ± 0.28 (0.95–2.16)	**0.006**
Isomaltose (%)	1.11 ± 0.40 (0.48–2.04)	0.84 ± 0.35 (0.31–2.15)	**0.004**
Melezitose (%)	0.75 ± 0.39 (0.09–1.46)	1.27 ± 0.84 (0.09–3.27)	**0.005**
Total sugars (%)	80.60 ± 2.80 (74.57–84.75)	82.80 ± 2.00 (77.73–85.98)	**0.000**
Glucose + Fructose	70.83 ± 2.32 (65.30–75.80)	72.82 ± 2.31 (66.90–78.40)	**0.001**
Fructose/Glucose ratio	1.28 ± 0.13 (1.07–1.51)	1.19 ± 0.08 (1.00–1.44)	**0.000**
Glucose/Moisture ratio	1.84 ± 0.16 (1.48–2.05)	1.99 ± 0.14 (1.72–2.25)	**0.000**
Maltose/Isomaltose ratio	7.04 ± 2.41 (2.88–13.88)	5.53 ± 1.80 (2.20–10.04)	**0.008**
Maltose/Turanose ratio	4.23 ± 0.89 (1.64–5.80)	3.89 ± 0.95 (2.32–6.99)	0.140
Sucrose/Turanose ratio	0.80 ± 0.59 (0.03–2.97)	0.37 ± 0.49 (0.02–1.97)	**0.003**

[a] Bold letters indicated that the differences between mean values were statistically significant ($p < 0.05$).

Electric conductivity was used to discriminate both types of honey, therefore logically the suspected honeydew honeys had a mean value higher than the blossom honeys. In general, the honeydew honeys analysed here were darker than the corresponding blossom honeys. A significant difference in the mean colour value was found between both types of honey, which has been observed by other investigators (González-Miret, Terrab, Hernanz, Fernández-Recamales, & Heredia, 2007; Marini et al., 2004; Terrab et al., 2003). It has been observed that the darkest coloured honeys presented the highest antioxidant capacity (McKibben & Engeseth, 2002). Besides which, the content of phenolic compounds has been associated with the darker colour commonly observed in honeydew honeys. Therefore, phenolic compounds must be partly responsible for the antioxidant effects of honeys, but other factors must obviously be involved (Vela et al., 2007).

HMF is an excellent indicator of honey freshness. Although the HMF content of the honeys should be under 40 mg/kg according to international trade guidelines (European Economic Community, 2002), some European bee federations differentiated some of their honeys as being "quality honey", when the HMF content was lower than 15 mg/kg (Bogdanov et al., 1999). Therefore, a maximum of 20 mg/kg was established for quality honeys produced in Tenerife according to the Quality Norm for Tenerife honeys. Most (73; 94.8%) of the honeys analysed had an HMF value lower than this limit, and none showed values higher than 28.4 mg/kg and so the honey samples analysed in this paper, in general, can be considered as fresh honeys. Suspected honeydew honeys had a higher HMF mean concentration than those found for blossom honeys, which agrees with results reported by other authors (Terrab et al., 2002; Turhan, Tetik, Karhan, Gurel, & Tavukcuoglu, 2008).

All the honeys analysed were acidic with a pH in the range of 3.52 and 6.91, and the free acidity had contents below 50 mEq/kg, which is three times the maximum established in the European legislation for this parameter (European Economic Community,

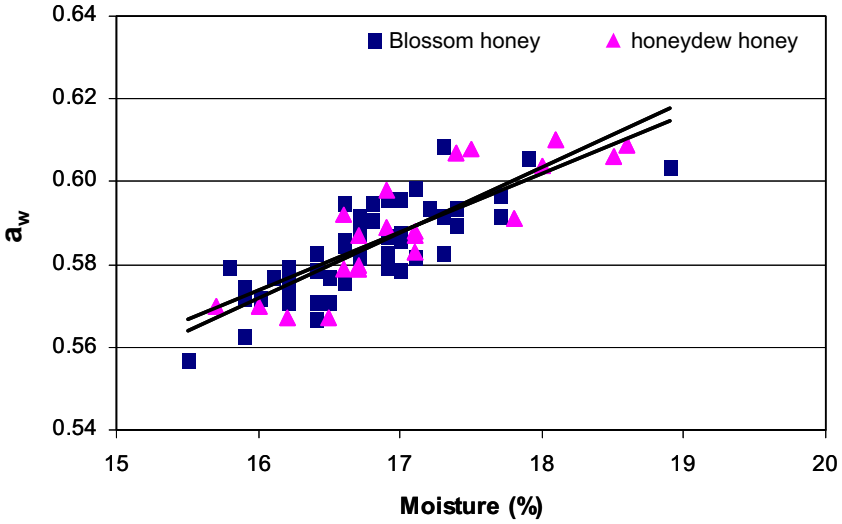

Fig. 1. Correlations between a_w and moisture according to the type of honey.

2002). Suspected honeydew honeys showed a higher mean pH value and acidity than blossom honeys, which agrees with data reported in the literature (Conti, Stripeikis, Campanella, Cucina, & Tudino, 2007; Marini et al., 2004; Ouchemoukh et al., 2007; Terrab et al., 2003; Turhan et al., 2008; Vela et al., 2007). The pH of the suspected honeydew honeys showed significant correlations with acidity ($r = -0.795$) and with HMF ($r = 0.695$), which were not observed in the blossom honeys. This demonstrates that both honeys behave differently with respect to these parameters.

Proline proceeds mainly from the salivary secretions of *Apis mellifera* during the conversion of nectar in honey. The content of proline is associated with the antioxidant capacity of honey (Meda, Lamien, Romito, Millogo, & Nacoulma, 2005). So, the correlation between radical scavenging activity and proline was higher than that correlation with total phenolic compounds. Suspected honeydew honeys showed a higher mean proline concentration than blossom honey, which agrees with the results of other authors (Ouchemoukh et al., 2007). However, a high variation in the concentration of proline was observed in the analysed honeys ranging between 309.7 and 1688 mg/kg, with a coefficient of variation of 38.9%. In accordance with Sánchez, Huidobro, Mato, Muniategui, and Sancho (2001), the high variability of the proline concentration made it impossible to characterise the origin (blossom or honeydew) of the honey. On the other hand, proline content has been used as a criterion of honey ripeness and, in some cases, of sugar adulteration. A minimum value of 180 mg/kg is accepted as the limit for genuine honey (Bogdanov et al., 1999). Therefore, in accordance with this, all the honeys analysed here are ripened and not adulterated.

Diastase and invertase activities are commonly used in Europe as a measure of honey freshness, because their activity decreases in old or heated honey. Diastase activity (°Gothe) varied between 8.9 and 35.9 and between 4.7 and 25.8 for the blossom and suspected honeydew honeys, respectively. These ranges are similar to those ranges found by Persano Oddo, Piazza, and Pulcini (1999). The invertase values (IU) were in the range of data reported for blossom honeys, however, the results corresponding to honeydew honeys were clearly lower than data in the literature (Persano Oddo et al., 1999). Invertase activity in honeydew honey was significantly higher than that found in blossom honey; however, no significant differences were observed in the diastase activity. Many investigators (Persano Oddo et al., 1999; Serrano, Espejo, Villarejo, & Jodral, 2007) have pointed out a highly significant and positive correlation between diastase and invertase activities; however, we have failed to obtain this significant correlation in all the honey samples, and in honeydew and blossom honeys independently.

Sugar composition has been used to discriminate honey samples by botanical origin (Cotte, Casabianca, Chardon, Lheritier, & Grenier-Loustalot, 2004) or geographical origin (Gómez Bárez et al., 2000). However, other investigators have concluded that sugar composition alone is not enough to discriminate honeys (Földhazi, 1994). Similar chromatograms were obtained for both types of honey considered. In both cases fructose, glucose, sucrose, maltose, trehalose, turanose, isomaltose and melezitose were identified and quantified. The monosaccharides glucose and fructose were clearly the predominant sugars, which confirm that all honey samples are genuine honeys. A good resolution and separation of the sucrose was also observed for both types of honey. Other sugars such as other disaccharides and trisaccharides were not well resolved. However, the chromatographic method used here was relatively fast (≅30 min) and an approximate sugar profile was obtained. Juszczak, Socha, Roznowski, Fortuna, and Nalepka (2009) have identified the same sugars in herb honeys, except for the fact they did not identify trehalose. In accordance with Juszczak et al. (2009), we did not identify other trisaccharides such as maltotriose or raffinose which were observed by other investigators in other honeys (Da Costa Leite et al., 2000; Ruiz-Matute, Ramos, Martínez-Castro, & Sanz, 2007). On the other hand, the peak corresponding to melezitose is probably a mix of melezitose and erlose. Therefore, some authors (Nozal, Bernal, Toribio, Alamo, & Diego, 2005; Ruiz-Matute et al., 2007) have managed to identify erlose and melezitose independently in natural honeys. Nozal et al. (2005) have separated 14 carbohydrates (including monosaccharides and oligosaccharides) using HPLC coupled to pulse amperometric detection. Low detection limits of the sugars can be achieved using this detector (Cataldi, Campa, & de Benedetto, 2000). However, the method is long and time consuming ≅60 min plus 30 min for cleaning and conditioning steps (Nozal et al., 2005).

The suspected honeydew honeys had significantly higher mean values of trehalose and isomaltose, and lower values of glucose, sucrose, turanose and melezitose, than the blossom honeys. Several authors (Golob & Plestenjak, 1999; Mateo & Bosch-Reig, 1997) found lower glucose and fructose contents in honeydew honeys than those contents found in blossom honeys. Golob and Plestenjak (1999) did not find significant differences in the mean sucrose concentrations between both types of honey from Slovenia. Our data of sucrose are in agreement with other data reported in

the literature (Mateo & Bosch-Reig, 1997) for several monofloral and honeydew honeys, except orange blossom that had a very high mean value of 4.4 ± 3.3%. All honey samples had a sucrose content lower than 5/100 g, which is generally taken as the limit value for honeys allowed by European Community Directive (European Economic Community, 2002). Apart from sucrose, other identified disaccharides were: maltose and isomaltose. Maltose ranged between 2.33 and 7.00/100 g, which agrees with results for honeys of other origins, reported by other authors (Juszczak et al., 2009; Mateo & Bosch-Reig, 1997). Terrab et al. (2003) gave a wider range 0.48–9.19/100 g for Moroccan honeys. The isomaltose concentrations were between 0.31 and 2.15/100 g, which was somewhat higher than other values (<1/100 g) reported by Juszczak et al. (2009). Suspected honeydew honeys showed a higher ($p < 0.05$) isomaltose concentration than blossom honey, which agrees with other authors (Mateo & Bosch-Reig, 1997). The trehalose concentration varied between 1.15 and 2.85/100 g, and higher ($p < 0.05$) concentrations were observed in the honeydew honeys. Melezitose has also been proposed to differentiate honeydew and blossom honeys (Persano Oddo & Piro, 2004). Suspected honeydew honeys had a lower mean melezitose concentration than that concentration found for blossom honeys.

Several carbohydrate ratios proposed to ascertain honey authenticity are also exposed in Table 2. Significant differences were observed in all the carbohydrate ratios between both types of honeys. Mean total sugar concentrations were 82.8 ± 2.0 and 80.6 ± 2.8/100 g for blossom and suspected honeydew honeys respectively, with significant differences between these values. These values are in agreement with data reported by other investigators (Conti et al., 2007; Terrab et al., 2003). All the honeys presented a value of glucose plus fructose higher than 60/100 g, which is the value required for all the kinds of honey in the European and Codex standards. In accordance with Soria, González, de Lorenzo, Martínez-Castro, and Sanz (2004) the sum of glucose plus fructose was a discriminatory variable used to distinguish between blossom and suspected honeydew honeys, which is due to the differences observed between the glucose content. However, this contrasted with the results reported by other investigators (Vela et al., 2007) who did not find significant differences between both types of honey.

Fructose/Glucose (F/G) ratio has been recommended to evaluate honey granulation because glucose is less water soluble than fructose (Ojeda de Rodríguez, Sulbarán de Ferrer, Ferrer, & Rodríguez, 2004). The proportion of fructose to glucose depends largely on the nectar source (Anklam, 1998). Blossom honeys contained more ($p < 0.05$) glucose than suspected honeydew honeys, therefore, the F/G ratio in blossom honey was lower than in honeydew honeys. Most investigators (Mateo & Bosch-Reig, 1997; Nozal et al., 2005; Ojeda de Rodríguez et al., 2004; Pérez-Arquillué, Conchello, Ariño, Juan, & Herrera, 1995) reported an F/G average ratio around 1.2, which coincides with our data for blossom honeys. Mateo and Bosch-Reig (1997) found a higher F/G ratio in honeydew honeys (1.33 ± 0.06) which agrees with the results observed here. Bauer (2001) however, found more marked differences, an F/G ratio of 1.0 and about 1.5–2.0 for blossom and honeydew honey, respectively.

In accordance with Manikis and Thrasivoulou (2001) the Glucose/Moisture ratio could be a better indicator for predicting honey crystallization. All the blossom honeys and most (20; 83%) of the suspected honeydew honeys had a value of this ratio higher than 1.7. When the glucose/water ratio is below this value, a slowly crystallizing honey is produced which is consistent with that observed in the honeys analysed here.

Furthermore, the intervals found in the case of the Sucrose/Turanose ratio were in agreement with those proposed in the bibliography (Horváth & Molnár-Perl, 1997). The Maltose/Isomaltose

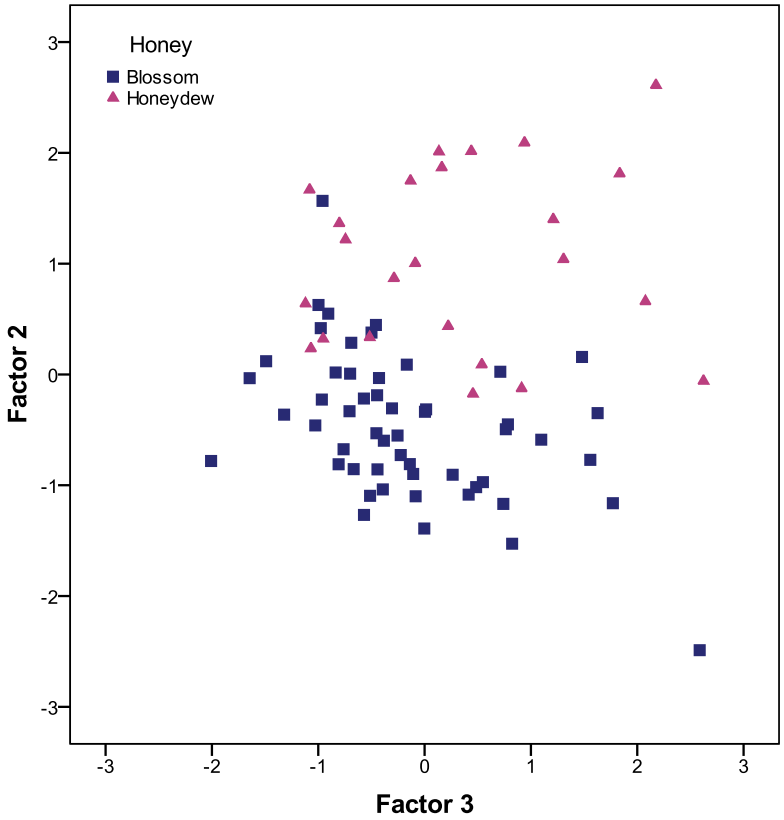

Fig. 2. Representation of the scores plot for all the honey samples on the second and third factor.

and Maltose/Turanose ratios were clearly higher than the intervals proposed (Horváth & Molnár-Perl, 1997), which is due to the high value of maltose. The presence of other sugars in the chromatographic peak of the maltose could explain the relatively high contents observed. However, our data on maltose were similar to the data reported by other researchers (Nozal et al., 2005). Thus, these proposed intervals should only be used as an orientation because it is possible that a natural honey could be falsely rejected. Therefore, the enormous diversity of natural honeys should be considered before making use of carbohydrate ratio intervals for testing natural honeys (Nozal et al., 2005).

3.3. Multivariate analysis

Multivariate analysis is being increasingly used for classifying groups of honeys when the classification using individual parameters is not successful. Factor analysis was applied to all the samples of honeys studied to establish a more simplified view of the relationship among the physicochemical and sugar parameters analysed. The first seven factors, accounting for 79.3% of the total variance, were chosen because their eigenvalues were higher than 1, and therefore, they account for more variance than the original variables. A Varimax rotation was conducted to minimise the number of variables influencing each factor facilitating the interpretation of the results. The first factor to explain the higher percentage of variance (25.9%) is strongly associated with turanose and, to a lesser extent, with HMF and acidity. Electric conductivity, and to some extent invertase and pH, were associated with the second factor. The third factor is related with isomaltose and inversely with glucose and total sugars; and the fourth factor with moisture and water activity. The fifth and seventh factors were inversely associated with melezitose and fructose respectively, with the sixth factor involving maltose. Fig. 2 shows the representation of the score plots of the second and third factors for all the honey samples. One can see that the honey samples belonging to the blossom honeys tend to differentiate from the samples of the suspected honeydew honeys. Although there is overlapping of some honey samples between the groups of honeys considered.

We also performed a linear discriminant analysis (LDA) to differentiate the honey samples according to both types of honey considered (blossom and suspected honeydew honeys). For obvious reasons, the electric conductivity was eliminated in this statistical study, since this variable has been used to classify the two types of honey. After application of the stepwise LDA to the data (Table 3), most of the honey samples (98.7% and 97.4% after cross-validation) were correctly classified selecting the following variables: pH, acidity, proline, diastase, invertase and melezitose. All blossom honeys were correctly classified and only one honeydew honey was erroneously included as blossom honey. When all the variables were introduced in the LDA, the percentage of correct classification was the same, 98.7% (96.1.0% after cross validation).

These results indicate that the two groups of honey previously classified according to electrical conductivity, had clearly different physicochemical and sugar characteristics which confirms that both types of honey could, in the main, be considered as honeydew and blossom honeys, respectively.

Table 3
Results of the stepwise discriminant analysis for all the samples considered to differentiate type of honey.

Honey	Number of sample and percentage (%) of correct classification	
	Blossom	Honeydew
Blossom	53 (100%)	0 (0%)
Honeydew	1 (4.2%)	23 (95.8%)

4. Conclusions

Considerable differences in the physicochemical parameters, sugar composition and palinological parameters between suspected honeydew and blossom honeys were observed. The application of multivariate analysis on the physicochemical parameters and sugar composition is a useful tool to differentiate these types of honey.

Acknowledgements

This work was financed by a research contract "Sugar determination for the characterisation of honeys from Tenerife" within the Convenio Marco between Exmo. Cabildo Insular and University of La Laguna to carry out scientific and technological studies related to water, agriculture, fisheries and food (Anexo n° 21). We also gratefully acknowledge the help of Patrick Dennis for revising and checking the English in this paper.

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